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The Expression and Regulation of STATs during 3T3-L1 Adipocyte Differentiation*

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STATs (Signal Transducers and Activators of Transcription) comprise a family of transcription factors that reside in the cytoplasm of resting cells. In response to a variety of stimuli, STATs become tyrosine-phosphorylated and translocate to the nucleus where they mediate transcriptional regulation. We have used the 3T3-L1 murine cell line to examine the expression of STAT proteins as a function of their differentiation into adipocytes. The expression of STATs 1, 3, and 5, but not of STAT 6, is markedly elevated in adipocytes as compared with their fibroblast precursors. Exposure of 3T3-L1 preadipocytes to tumor necrosis factor α (TNFα) blocks their differentiation into adipocytes. Therefore, we examined STAT expression as a function of differentiation in the presence of this cytokine. The expression of STATs 1 and 5 is markedly attenuated in the presence of TNFα, whereas STAT 3 expression is unaffected by this treatment. Only STAT 1 is down-regulated by TNFα in fully differentiated cells. Thus, although the expression of STATs 1, 3, and 5 is markedly enhanced upon differentiation, only STAT 5 expression is tightly correlated with the adipocyte phenotype. These data suggest that STAT 5, and possibly STAT 1, could be potential inducers of tissue-specific genes, which contribute to the development and maintenance of the adipocyte phenotype.

The 3T3-L1 cell line differentiates under the controlled conditions of cell culture from fibroblasts, or preadipocytes, into cells with the morphological and biochemical properties of adipocytes (Green and Kehinde, 1974; Green and Kehinde, 1976) in a process that closely resembles the development of adipose tissue *in vivo*. Upon differentiation, these cells acquire sensitivity to hormones and exhibit a coordinate increase in the activities of numerous enzymes in the lipolytic, lipogenic, and glycolytic pathways (Smas and Sul, 1995). To date, members of two transcription factor families, C/EBP (C/AAAT Enhancer Binding Proteins) and PPAR (Peroxisome Proliferator Activated Receptors) have been shown to be induced during adipocyte differentiation and are thought to play a significant role in the regulation of fat-specific gene expression.

The STAT (Signal Transducers and Activators of Transcription) family of transcription factors is comprised of six family members (STATs 1–6) that, in response to stimulation of various receptors, mainly those for cytokines, are phosphorylated on tyrosine residues, which causes their translocation to the nucleus. Each STAT family member shows a distinct pattern of activation by cytokines, has a unique tissue distribution, and upon nuclear translocation can regulate the transcription of particular genes (Schindler and Darnell, 1995; Ihle, 1995). The likely order of events for STAT activation can be described as follows: 1) ligand binding of cell surface receptor; 2) receptor association with a JAK (Janus kinase) kinase family member; 3) JAK tyrosine phosphorylation of STAT proteins; 4) dimerization of the STATs; 5) translocation to the nucleus; and 6) DNA binding. STATs have been shown to bind at least three different consensus sequences, and this binding regulates the transcription of specific genes (Schindler and Darnell, 1995; Ihle, 1995).

One of the first identified inhibitors of adipocyte differentiation was tumor necrosis factor-α (TNFα),[1] a cytokine that elicits a wide range of biological effects including the regulation of growth and differentiation. In addition, TNFα has been shown to down-regulate the insulin responsiveness of fully differentiated adipocytes (Stephens and Pekala, 1991; Hotamisligil *et al.* 1993). Because regulation of the STATs is mainly cytokine-mediated, TNFα could be a mediator of STAT expression during and/or after adipocyte differentiation. Most of the studies on the STAT family of transcription factors have focused on their tyrosine phosphorylation and DNA binding. In this report, we demonstrate that another level of regulation of these proteins exists as they are induced during the differentiation of adipose cells in culture. Moreover, we demonstrate that inhibition of differentiation by TNFα completely suppresses the expression of two STAT family members. We interpret these data to indicate that STAT family members may play a role in the regulation of genes that contribute to the phenotype of the mature adipocyte.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (Cornelius *et al.*, 1990). Briefly, cells were plated and grown to 2 days postconfluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum. Differentiation was then induced by changing the medium to DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 48 h, the differentiation medium was replaced with maintenance medium containing DMEM supplemented with 10% FBS. The maintenance medium was changed every 48 h until the cells were utilized for experimentation. Human recombinant TNFα (Quality Control Biochemical) was resuspended in phosphate-buffered saline containing 0.1% fatty acid-free and growth factor-depleted bovine serum albumin (Sigma).

Rat Adipocytes—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion as described (Rodbell, 1964). Cell fractionation was performed as described previously (Simpson *et al.*, 1983), and the cytosol fraction was

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[1] The abbreviations used are: TNFα, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; C/EBP, C/AAAT enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; PAGE, polyacrylamide gel electrophoresis.

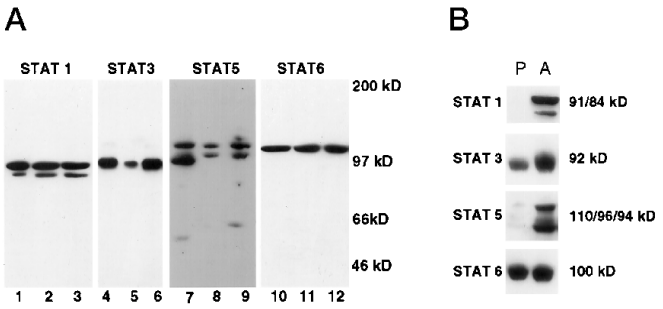

FIG. 1. **The expression of STATs in adipocytes.** *Panel A*, whole cell extracts were prepared from 3T3-L1 adipocytes and the cells listed below. In addition, cytosolic extract was prepared from the adipocytes of rat epididymal fat pads. Positive controls for STAT immunoblotting were provided by Transduction Laboratories and were as follows: A431 cells for STAT 1, human fibroblasts for STAT 3, RSV-3T3 mouse fibroblast cell line for STAT 5, and Jurkat cells derived from acute T-cell leukemia for STAT 6. Cell extracts from 3T3-L1 adipocytes (*lanes 1, 4, 7,* and *10*) and cytosol from rat epididymal fat (*lanes 2, 5, 8,* and *11*) were divided and blotted simultaneously, while a different positive control (*lanes 3, 6, 9,* and *12*) was used to examine the expression of STAT family members. The whole gel for each STAT family member is shown in *panel A*, while the remainder of the figures only includes the part of the blot that had a signal as these antibodies do not have any cross-reactivity as shown in this panel. *Panel B*, whole cell extracts were isolated from growing 3T3-L1 preadipocytes (*P*) and fully differentiated 3T3-L1 adipocytes (*A*). In each panel, 50 μg of each preparation were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The detection system was horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence substrate kit.

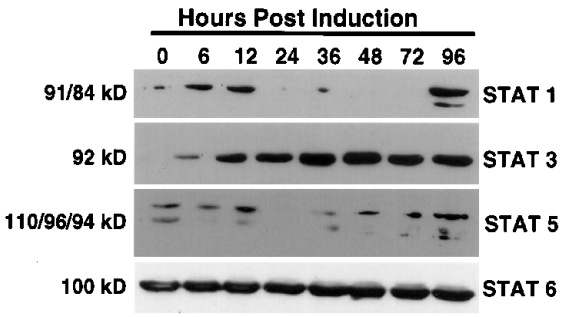

FIG. 2. **Early induction of STATs during 3T3-L1 differentiation.** Whole cell extracts were prepared from 3T3-L1 cells at various times following the induction of differentiation. Cells were induced to differentiate at 2 days postconfluence with the addition of a differentiation mixture containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 48 h this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this condition thorough the remainder of the analysis. Samples were processed and results were visualized as described in Fig. 1.

used for Western blotting.

Preparation of Whole Cell Extracts—3T3-L1 cells were rinsed with phosphate-buffered saline and then harvested in a buffer containing 25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, and 10 μM leupeptin. Protein content for whole cell extracts and rat cytosol were determined using a BCA kit (Pierce) according to the manufacturer's instructions.

Gel Electrophoresis and Immunoblotting—Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing SDS according to Laemmli (Laemmli, 1970) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 h at room temperature. The STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories. Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

RESULTS

The expression in adipocytes of the various STAT proteins, as detected by Western blot, is depicted in Fig. 1. The profile in *panel A* illustrates that STAT 1, STAT 3, STAT 5, and STAT 6 have similar levels of expression in the whole cell extracts from 3T3-L1 cultured murine adipocytes (*lanes 1*, *4*, *7*, and *10*) and from the cytosol of rat epididymal fat cells (*lanes 2*, *5*, *8*, and *11*). The *third lane* of each *panel* shows STAT expression in cellular extracts (provided by Transduction Laboratories) from cells known to express these proteins at substantial levels (*lanes 3*, *6*, *9*, and *12*). It can be seen that STAT expression in adipocyte extracts was equivalent to or greater than cells known to express these proteins. The expression of STAT 4 protein was undetectable in either cultured adipocytes or adipose tissue when compared with positive controls (data not shown), and species-specific antibodies for STAT 2 are not commercially available at this time. *Panel A* also illustrates that the 91- and 84-kDa proteins of STAT 1, reported as alternatively spliced products of the same gene (Schindler *et al.* 1995), were detectable in both cultured adipocytes and adipose tissue. The monoclonal antibody for STAT 5 reacted with three protein products from cultured adipocytes (best illustrated in Fig. 3). The 96- and 94-kDa doublet has been consistently identified in various other cell types and postulated to be alternatively spliced gene products of STAT 5 (Schindler and Darnell, 1995). The higher molecular mass band at 110 kDa has been speculated to be a phosphorylated form of STAT 5 (Barahmand-pour *et al.*, 1995). Alternatively, this protein product could be another form of STAT 5 or even an unidentified STAT family member. Monoclonal antibodies for STAT 3 and STAT 6 reacted with single protein products with molecular masses of 92 and 100 kDa, respectively.

Panel B of Fig. 1 depicts the expression of STAT proteins in 3T3-L1 cells before and after differentiation into adipocytes. As previously reported, the fully differentiated phenotype is attained 6–8 days following the addition of an induction mixture to the cell medium of postconfluent preadipocytes (Green and Kehinde, 1974, 1976). As illustrated in *panel B*, STAT 1 and STAT 5 proteins were dramatically elevated after differentiation, and STAT 3 was expressed at a greater level in adipocytes as compared with preadipocytes, whereas STAT 6 was unchanged.

The correlation of STAT protein expression with adipocyte differentiation is further depicted in Figs. 2 and 3 where the protein amounts were measured during the time course of differentiation. As shown in Fig. 2, STAT 1, STAT 3, and STAT 5 proteins were minimally expressed in preadipocytes (0 h), similar to that illustrated in *panel B* of Fig. 1. The level of all three STAT proteins was increased 6–12 h following the addition of the differentiation mixture to the cell medium of postconfluent preadipocytes. STAT 3 protein expression plateaued approximately 36 h following the induction of differentiation and remained at this level throughout the time course. On the other hand, STAT 1 and STAT 5 protein amounts decreased between 24 and 72 h, then increased by 96 h, and remained significantly elevated over the amount of protein expressed in preadipocytes. This transient down-regulation of STAT 1 and STAT 5 was observed in four independent experiments. The amount of STAT 6 did not vary as a function of differentiation.

Fig. 3 illustrates STAT protein expression over a 7-day period, which was sufficient to allow for the development of the fully differentiated adipocyte phenotype. This time course was also performed in the presence of TNFα, a cytokine known to inhibit adipose conversion as judged by triacylglycerol accumulation and inhibition of expression of fat-specific genes (Torti *et al.*, 1989). As in the previous figures, STAT 1, STAT 3, and STAT 5 protein amounts increased with time, while STAT 1 and STAT 5 were transiently decreased during the early

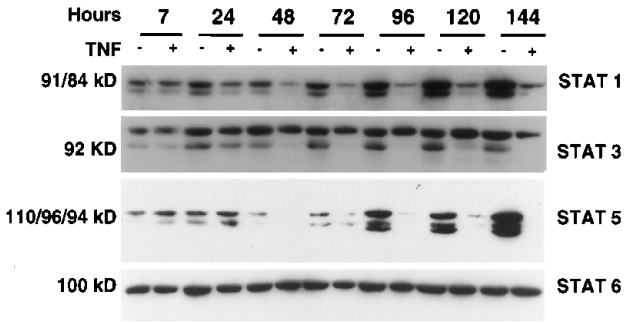

FIG. 3. **STAT expression during 3T3-L1 differentiation (−/+ TNFα).** Whole cell extracts were prepared from 3T3-L1 cells at various times following the induction of differentiation in the presence and absence of TNFα. Cells were induced to differentiate as described in Fig. 2, except that TNFα (1 nM) was added to the differentiation mixture of some cells. Fifty μg of protein were separated by SDS-PAGE, transferred to nitrocellulose, subjected to Western blot analysis, and visualized as described in Fig. 1.

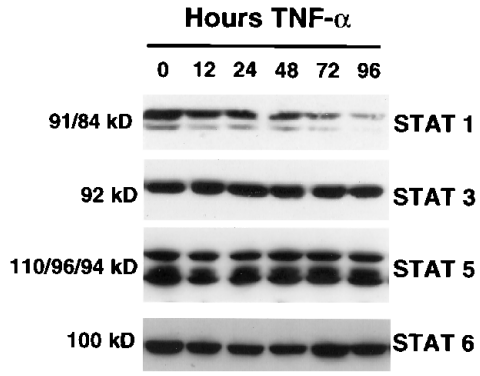

FIG. 4. **The regulation of STAT expression by TNFα in 3T3-L1 adipocytes.** Fully differentiated 3T3-L1 adipocytes were exposed to 1 nM TNFα for various times, and then whole cell extracts were isolated and an equal amount of proteins was separated by SDS-PAGE, transferred to nitrocellulose, subjected to Western blot analysis, and visualized as described in Fig. 1.

phases of differentiation. The lower molecular mass protein present in the STAT 3 panel likely represents cross-reaction with the 84-kDa protein of STAT 1 (Bonni *et al.*, 1993). Of particular interest, Fig. 3 shows that inhibition of differentiation by TNFα completely obliterated the increase in STAT 1 and STAT 5 expression without affecting STAT 3. Again, STAT 6 expression was unaffected under all conditions.

The suppression of STAT 1 and STAT 5 expression could be due to a direct down-regulation by TNFα rather than by the events involved in the differentiation process. To test for this possibility, 3T3-L1 adipocytes were fully differentiated (8 days after the induction of differentiation) and then treated with TNFα over a time course known to effect the regulation of genes such as the insulin-sensitive glucose transporter (GLUT4) and the fat-specific lipid binding protein (aP2/422) (Stephens and Pekala, 1991; Stephens and Pekala, 1992). Fig. 4 illustrates that treatment of fully differentiated adipocytes with TNFα resulted in a specific and significant decrease in STAT 1 protein levels. After 96 h exposure to TNFα, there was a 90% decrease in STAT 1 expression whereas STATs 3, 5, and 6 were completely unaffected by this treatment.

DISCUSSION

Members of the STAT family have been well documented to regulate gene expression following their activation by cytokines and other stimuli (Schindler and Darnell, 1995; Ihle, 1995). Moreover, the tissue distribution of each STAT is unique suggesting that the regulation of tissue-specific genes may be a physiological role for these proteins. Here, we provide indirect support for this hypothesis by demonstrating that STATs 1, 3, and 5 are induced during the differentiation of 3T3-L1 cells from fibroblasts to adipocytes. This expression does not appear to be an artifact of the cell culture system, as these same proteins are readily detectable in rat adipose cells. The induction of these family members during adipocyte differentiation indicates that this family of transcription factors can be regulated at the level of their expression as well as by their cytokine-mediated phosphorylation and nuclear translocation.

To date, two families of transcription factors, the C/AAAT enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPARs), have been shown to be induced during adipocyte differentiation and to play a significant role in the regulation of fat-specific genes. C/EBPα is induced late (60–96 h) during 3T3-L1 adipocyte differentiation and has been shown to regulate the transcription of a number of fat-specific genes (Cornelius *et al.*, 1994). Expression of C/EBPα in fibroblast cell lines can promote adipogenesis (Freytag *et al.*, 1994), while expression of C/EBPα antisense RNA blocks the differentiation of 3T3-L1 adipocytes and the expression of some fat-specific genes (Lin and Lane, 1992). PPARγ is a recently cloned member of the peroxisome proliferator-activated receptor family and has been identified as a component of the adipogenic transcription factor complex (ARF6), which regulates transcription of the fat-specific gene aP2/422 (Tontonoz *et al.*, 1994a). PPARγ is expressed primarily in adipocytes and is induced very early in the process of adipocyte differentiation. Like C/EBPα, when ectopically expressed in a number of fibroblast cell lines, PPARγ can induce adipogenesis (Tontonoz *et al.*, 1994b). We show here that three members of the STAT family of transcription factors, STATs 1, 3, and 5, are induced during differentiation in a manner similar to C/EBPα and PPARγ. Therefore, these STATs could potentially play a critical role in both the development of the adipose phenotype and the regulation of expression of fat-specific genes.

Since STAT 1 and STAT 5 are highly induced during differentiation and their accumulation is repressed when differentiation is inhibited, it is likely that these STAT family members could be transcriptional regulators involved in the development and/or maintenance of the adipose phenotype. However, the repression of STAT 1 and STAT 5, which occurs when inhibiting differentiation with TNFα, could be due to a direct effect of TNFα on STAT expression. In fact, this may be the case for STAT 1 whose expression is severely down-regulated when fully differentiated 3T3-L1 adipocytes are exposed to prolonged TNFα treatment (Fig. 4, see also, next paragraph). TNFα has no effect on STAT 5 accumulation in fully differentiated adipocytes (Fig. 4), and STAT 5 expression strongly correlates with the degree of adipocyte differentiation when this process is manipulated by subtraction of differentiation mixture elements (data not shown). STAT 3 expression is increased upon conversion of preadipocytes to adipocytes (Fig. 2), but its induction is not inhibited with TNFα, which also inhibits differentiation (Fig. 3), thus suggesting that its increased expression is unrelated to this process. However, STAT 3 is still likely to have a function in the terminally differentiated adipocyte as it is clearly present in the fully differentiated adipocytes and in rat fat cells (Fig. 1). STAT 1 expression also correlates with the degree of adipocyte differentiation, albeit to a lesser extent than STAT 5 (data not shown).

The transient down-regulation of STAT 1 and STAT 5 during differentiation occurs between 24 and 72 h after the induction of differentiation. This time frame overlaps with the presence of the differentiation-inducing mixture (0–48 h), and it is possible that the combination of hormones present in the mixture may be responsible for this temporary down-regulation of

STAT 1 and STAT 5 protein levels, either directly or indirectly, by inducing the expression of some additional effector (inhibitor) of STAT expression. We are currently examining the effects of specific components of the induction mixture on STAT expression during differentiation.

Exposure of fully differentiated adipocytes to TNFα results in a highly specific and significant decrease in STAT 1 expression (Fig. 4). As previously shown, this exposure to TNFα did not result in dedifferentiation or a loss of lipid content (Stephens and Pekala, 1991). Recent studies have demonstrated that TNFα treatment of fully differentiated cultured adipocytes results in insulin resistance, which is accompanied by the down-regulation of the insulin-sensitive glucose transporter (GLUT4) and the insulin receptor (Stephens and Pekala, 1991; Hotamisligil *et al.*, 1993). Furthermore, the observed decrease in STAT 1 parallels the TNFα-induced repression of GLUT4.[2] Given that the induction of STAT 1 expression is concomitant with the acquisition of insulin sensitivity of 3T3-L1 adipocytes and is down-regulated by TNFα in a condition of insulin resistance, we hypothesize that STAT 1 expression and function may be contributing to the regulation of genes involved in insulin sensitivity in 3T3-L1 adipocytes. We are in the process of experimentally addressing this and other hypotheses concerning the physiological role of STATs 1, 3, and 5 in adipocyte differentiation and gene expression.

[2] J. M. Stephens, R. F. Morrison, and P. F. Pilch, unpublished observation.

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